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SUMMARY

1. PURPOSE. To provide security and policy review on the document at Tab 1 prior to release to the public.

2. BACKGROUND.

Authors: Hallenbeck PC (LSRC/USAFA), Grogger M (LSRC/USAFA), Mraz M (LSRC/USAFA), Veverka D (LSRC/USAFA).

Title: Building a Better Mousetrap I: using Design of Experiments with unconfounded ions to discover superior media for growth and lipid production by Chlorella sp. EN1234.

Release Information: This manuscript will be submitted for consideration for presentation in the journal Bioresource Technology. DOD disclaimer is located on the last page in Acknowledgements section.

3. DISCUSSION. N/A

4. VIEWS OF OTHERS. N/A

5. RECOMMENDATION. Sign coord block above indicating document is suitable for public release. Suitability is based solely on the document being unclassified, not jeopardizing DoD interests, and accurately portraying official policy.

Lt Col Ryan W. Maresh
Assistant Professor of Biology

Tab
1. Copy of manuscript

Elsevier Editorial System(tm) for Bioresource Technology
Manuscript Draft

Manuscript Number:

Title: Building a Better Mousetrap I: using Design of Experiments with unconfounded ions to discover superior media for growth and lipid production by Chlorella sp. EN1234

Article Type: SI: Bio/Chemicals from Algae

Keywords: Biofuels; algae; medium optimization; lipid production; unconfounded ion matric; Scheffe mix process

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Abstract: An unconfounded Scheffe Mix approach was used to probe important ions and their interactions in supporting biomass and lipid production by Chlorella sp. EN1234. Six major cations and anions; NH4+, NO3-, Na+, K+, PO4- and Cl-; were investigated. Piepel plots and RSM analysis showed that in a number of cases, the major media anions PO4- and Cl- negatively influence final cell densities, and that maximal cell density is obtained with nitrate over ammonium, with an optimal effect when mixed with equal molar potassium. Although it is commonly assumed that lipid content increases in nitrogen-deficient media, here little correlation was found between nitrogen content and total lipid content with mixtures that supported high lipid productivity. Thus these mixtures define the composition space within which further R&D might produce the best trade-off between total biomass production and high cellular lipid content.

Dear Editor,

We hereby submit to you our manuscript for consideration for publication in Bioresource Technology in the Special Issue: Algal Biofuels and Chemicals. With this letter I would like to attest to the following points:

1. This manuscript should be classed in the section Biofuels from Algae: 30.060

2. That all the authors mutually agree for submitting their manuscript to BITE

3. The manuscript is the original work of the authors,

4. The manuscript has not been submitted earlier to BITE

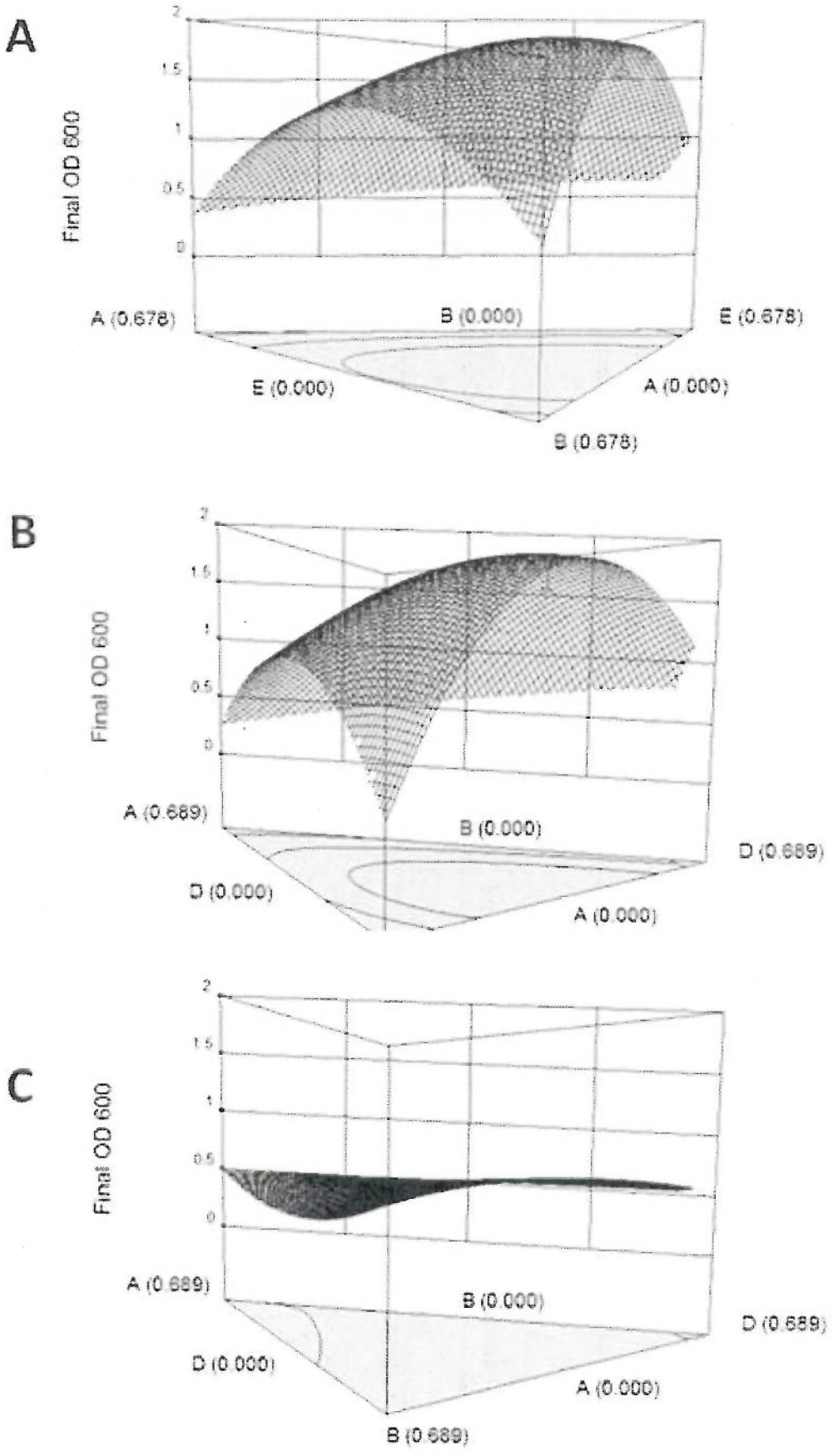
5. The manuscript is novel in several respects. It describes the use of DOE (design of experiments) to investigate the effects of media components on biomass and lipid production in a way that is very seldom used, an unconfounded ion study. Previous media composition studies have varied ions as their salts, leading to situations where it is impossible to determine if the effect observed is due to the ion of interest, or its counter ion, or simply the ionic strength. This pitfall is avoided in the present study. In addition to determining unique compositions that lead to high levels of biomass and lipid production, we have also investigated several novel aspects. For example, we show that chloride ion is a generalized inhibitor, and how different mixes of sodium and potassium might be useful depending upon the nitrogen source utilized.

I hope that you find our manuscript satisfactory for publication.

Sincerely,

Patrick C. Hallenbeck

Graphical Abstract (for review)



Highlights:

- An unconfounded mix approach was used to probe important ions & their interactions
- The major media anions $PO_4^-$ and $Cl^-$ negatively influence final cell densities
- With *Chlorella* EN1234, maximal cell density is obtained with nitrate
- Little correlation was found between nitrogen content and total lipid content
- A composition space is defined for the best trade-off in lipid production

# Building a Better Mousetrap I: using Design of Experiments with unconfounded ions to discover superior media for growth and lipid production by *Chlorella sp.* EN1234 [a]

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[a]*The views expressed in this article are those of the authors and do not reflect the official policy or position of the United States Air Force, the Department of Defense, or the US Government.*

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An unconfounded Scheffe Mix approach was used to probe important ions and their interactions in supporting biomass and lipid production by *Chlorella sp.* EN1234. Six major cations and anions; $NH_4^+$, $NO_3^-$, $Na^+$, $K^+$, $PO_4^-$ and $Cl^-$; were investigated. Piepel plots and RSM analysis showed that in a number of cases, the major media anions $PO_4^-$ and $Cl^-$ negatively influence final cell densities, and that maximal cell density is obtained with nitrate over ammonium, with an optimal effect when mixed with equal molar potassium. Although it is commonly assumed that lipid content increases in nitrogen-deficient media, here little correlation was found between nitrogen content and total lipid content with mixtures that supported high lipid productivity. Thus these mixtures define the composition space within which further R&D might produce the best trade-off between total biomass production and high cellular lipid content.

**1.0 Introduction**

Microalgae are a very diverse group of organisms which display a wide spectrum of potential ability to produce biofuels, neutraceuticals, or other valuable chemicals. In particular, they are under intense study recently for their potential for the sustainable production of biofuels (Abdelaziz et al., 2013 a, b; Fields et al., 2014; Leite & Hallenbeck 2011; Leite et al., 2013; Moody et al., 2014; Lee et al., 2014; li et al., 2014 ). Variation in microalgal capabilities is linked to the presence and diversity of metabolic pathways. In addition, nutrient composition and ratios might also determine where the cellular metabolism is directed. For example, key nutrients that reportedly affect lipid yield include carbon (in the form of carbon dioxide), nitrogen and phosphorus (Converti et al., 2009; Gordillo et al., 1998; Griffiths et al., 2014 a, b; Sheehan et al., 2008). Algal culture media

are composed of mineral nutrients in the form of salts and a great deal of effort over the past decade has been devoted to finding the optimal concentrations of salts for different species, resulting in a vast number of varying media recipes.

A number of studies have shown that microalgal cellular lipid accumulation, generally in the form of triacylglycerides, is positively affected by stress conditions such as nutrient deficiency or high irradiance (Harwood & Guschina, 2009; Hu et al. 2008; Roessler 1990). Due to the wide diversity of microalgal species and the complexity of cellular biochemical processes involved in carbon flux, it might be expected that there are general mechanisms for regulation of carbon flux that apply to all algae, as well as other, more specific regulatory processes that only apply to specific subgroups. Although many studies have focused on identifying the regulatory mechanisms for lipid accumulation under stress, relatively little is known about the correlation between lipid productivity of microalgae and general growth conditions. Until now, most strains of microalgae have been grown in cultivation media that were developed several decades ago for the general growth of larger groups of algae.

Changes in growth conditions; light intensity (Converti et al., 2009; Xia et al., 2013), temperature (Roleda et al., 2013), pH (Skrupski et al., 2013) and/or nutrient composition (Hu et al., 2008; Leite et al., 2013); can impact both lipid quality and quantity (positively or negatively). For example, a decrease in temperature, while not necessarily affecting the overall lipid yield, changes the lipid composition towards polyunsaturated fatty acids that are less suitable for biodiesel production (Converti et al., 2009). While irradiance can affect lipid production, high irradiance can actually reduce algal growth rate by

photoinhibition (Radakovits et al., 2010) and thereby reduce lipid productivity overall. These cellular responses to irradiance and temperature changes are largely species-dependent (Guschina et al., 2006).

Previously, nutrient manipulations aimed at changing the concentration of a particular ion have been achieved mainly by changing the amounts of particular salt constituents of the growth medium, neglecting the effect of the corresponding counter ion (Niedz and Evens, 2007). This leads to a confounding effect where it is not possible to quantitatively separate the effects due to the two different ions. As such, the total ion concentration is often increased in these studies, creating yet another series of confounded variables. In previous work, an experimental design approach was used to investigate the effects of five different ions on specific growth rates using a DOE (Design of Experiments) approach with an unconfounded ion matrix (Evens and Niedz, 2010). This type of experimental design was shown to produce a much richer data set than possible with a one-factor-at-a-time approach. The optimization of biofuel production is essential to making renewable fuel sources economically viable. Here this approach was used to examine the effects of media composition and other environmental manipulations on biomass and lipid accumulation with the ultimate goal of formulating a model that identifies the drivers for growth and lipid production and predicts the optimal media composition for desired productivity indices.

## 2.0 Materials and Methods

### *2.1 Strains and growth conditions*

*Chlorella sp.* EN1234 was obtained from Dr. Juergen Polle, Brooklyn College, and cultures were grown in 3 mL volumes in 12-well covered plates without agitation at 25°C, with a light intensity of 150 μE on a 16-hour light/8-hour dark cycle. Each 3 mL culture was inoculated with 3,000 algae cells/μL.

*2.2 Growth, lipid and biomass measurements*

Optical density (OD) measurements were taken at 600 nm with a BioTek μQuant® microplate spectrophotometer. Prior to measurement of OD measurement, cultures were agitated on a microtiter plate shaker for 2 minutes to re-suspend any settled algal cells. Variation between runs was ± 25% and variation between biological duplicates within the same run were ± 10%. Cell counts and neutral lipid productivity were determined using flow cytometry with a BD Accuri® C6 Flow Cytometer. Flow cytometry samples were prepared in 1.5 mL conical bottom tubes with 50 μL of culture and 150 μL of deionized water (final sample volume of 200 μL). Samples were measured without BODIPY® 493/503 (Life Technologies, D3922) dye and with 1 μL of 200X BODIPY® (200 ng/μL in DMSO). Dyed samples were vortexed and incubated for 10 minutes before measuring. Undyed samples were vortexed and measured immediately.

*2.3 Design of Experiments study of operational parameters*

Using experimental design software (Design Expert 8) and the mixture and optimal design functions, a matrix was developed to optimize freshwater media. Such mixture designs give response surface designs that are constrained such that all the component proportions add up to 1. Here, a Scheffe Mix Model with a quadratic process order, special cubic mix order and a sixth combined order limit was used. 210 media variations were generated and

used to assess algal biomass and neutral lipid production. Each medium consisted of a basal medium to which were added varying amounts of $H_3PO_4$, HCl, $HNO_3$, KOH, NaOH, $NH_4OH$, $Mg(NO_3)_2$ x $6H_2O$, $MgCl_2$ x 6 $H_2O$, and $Mg(OH)_2$ (Table S1).

*2.4 Phylogenetic analysis of EN1234*

EN1234 is a natural isolate that has been little characterized or described until now. To better understand its relationship to previously described microalgae, portions of its DNA encoding 18S rRNA and 23S rRNA were amplified by PCR and sequenced. Briefly, samples were held at 94°C for 5 minutes; subjected to 30 cycles of 94° for 40 seconds, 61°C for 40 seconds, and 72°C for 90 seconds; and then held at 72°C for 5 minutes before storing at 4°C. PCR reactions were run on an Eppendorf 6325 Mastercycler® Pro S PCR machine using an Ambion AgPath-ID One-Step RT-PCR® Kit (catalog # AM1005). PCR solution components included: 12.5 µL of 2X RT-PCR Buffer, 1 µL 5X RT-PCR Enzyme Mix, 1 µL of forward primer, 1 µL of reverse primer, 3 µL of algal culture, and 6.5 µL of nuclease-free water (total volume 25 µL). The primers used were: 23S (PRIMER_23SU-1 AGGGGTARAGCACTGYTTYG, PRIMER_23SU-2 CCTTCTCCCGAAGTTACG) and 18S (Forward: GTGGTAACGGGTGACGG, Reverse: GTGCGGCCCAGAACATC). The sequences obtained have been deposited in GenBank (accession numbers: KM213393, 23S, KM213394, 18S). Basic BLAST (NCBI) was used to obtain sequences showing significant homology. These were aligned online using CLUSTALW (http://www.genome.jp/tools/clustalw/) and the FASTA output was then used as input to tree construction using the Maximum Likelihood function of MEGA6 (http://www.megasoftware.net/).

## 3.0 Results and Discussion

### *3.1 Growth of Chlorella sp. EN1234 on the 210 solutions*

En1234 was originally supplied to us as a potential candidate for lipid production studies. Its phylogenetic relationship to previously studied algae was determined using 18S rRNA and 23S rRNA sequence information obtained as described in Materials and Methods. 18S rRNA sequences showed that it was highly similar to known *Chlorella* species (not shown). The results based on its plastid 23S rRNA sequence, which is more variable than that of the nuclear-encoded 18S rRNA, show that EN1234 is an outlier to known *Chlorella* species, most certainly within the family Oocystaceae and quite possibly a new *Chlorella* (Figure 1). For the purposes of this article it will be called *Chlorella* sp. EN1234.

### *3.2 Piepel plot analysis of the growth of Chlorella sp. EN1234 on the different mixtures*

Here the growth of this strain was assessed using the matrix of solutions set up as described in Materials and Methods. The final $OD_{600}$s obtained for *Chlorella sp.* EN1234 growing on the 210 solutions were fit to a special cubic (mix) linear (process) model. Anova (Table S2) gave a model F-value of 8.18 implying that the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. There appeared to be a good fit in terms of other parameters as well, as shown by a normal plot of the residuals and plots of residuals versus predicted and predicted versus actual (Figure S1). The variation in response ($OD_{600}$) expected for changes in single variables while the proportions of the others are held constant was examined using Piepel plots (Figure 1). These are trace plots which allow all the varied components to be viewed at once.

Essentially, the factors tool of the software sets the reference blend through which the traces are plotted, allowing one to visualize how sensitive the formulation is to deviations from the central point (reference blend). Thus, trace plots (also called perturbation plots in response surface and factorial designs) help one to compare the effects of all the components in the design space. In the Piepel plot variation used here, the component under study is varied, while the ratios of the other components are held constant.

When examined using Piepel plots (Figures 2 and 3), the mixtures under study here showed a number of salient points. Firstly, the predicted final cell density ($OD_{600}$) appeared to be sensitive to total ionic composition with the higher total ions (Fig. 2A) giving a higher OD than the lower ionic compositions (Fig. 2B and 2C). Secondly, as can be seen from the figure, at the lowest ionic concentration (2 mM) there is relatively little variation with changes in any of the parameters (Fig 2C), whereas all the changes are accentuated at 20 mM total ions (Fig. 2A). In all cases, $PO_4^-$ (C) and $Cl^-$ (F) are seen to negatively influence cell density in these mixtures over a relatively small range. With cultures at 20 mM total ions, $NH_4^+$ and $NO_3^-$ appear to have a negative impact over a longer range (i.e. to higher concentrations).

It was also interesting to examine the Piepel traces obtained with this model with *Chlorella sp.* EN1234 under the conditions that gave the highest response, run 44 (Figure 3). Again, these plots show that the greater the total ion concentration, the greater the final $OD_{600}$ that is obtained (Fig. 3A). These plots suggest that these conditions are optimal since variation of any of the parameters, either to greater or lesser amounts, leads to a drop-off in cell density. In this case, and especially at 20 mM total ions (Fig. 3A), changes in all

of the parameters had the same magnitude effect over most of the range visualized (Except for C and F which do not extend into the negative area since they are already at a minimum).

*3.3 RSM plot analysis of the growth of Chlorella sp. EN1234 on the different mixtures*

While Piepel plots can show roughly how the system as a whole responds, more detailed information about specific interactions between ions can be obtained from surface plots. For this purpose, surface plots under conditions where maximal $OD_{600}$s are obtained were examined for a few ions that provided insights (Figures 4 and 5). When mixtures of ammonium, nitrate, and potassium are varied at 20 mM total ions (Fig. 4A), a number of interesting effects are seen. Maximal cell density is seen with nitrate and little or no ammonium (along the AB axis) but the optimal effect of nitrate is seen when mixed with an equal amount of potassium ($Na^+$ constant at 0.312). With no nitrate present (axis AE) very little effect of mixing different proportions of potassium and ammonium is seen, thus this effect is specific to nitrate among the two nitrogen sources. Moreover, ammonium without any nitrate present only poorly supports growth.

Interestingly, when potassium is absent (axis AB) maximum growth is seen with an equal mixture of ammonium and nitrate. On the other hand, very similar analogous effects are seen if potassium is held constant (0.301) and ammonium, nitrate and sodium are varied at 20 mM total ions (Fig. 4B). Thus, as before, nitrate without any ammonium supports maximum cell density, but only in an equal mixture with sodium (BD axis). Again, in analogy with what was found with potassium, in the absence of sodium, an equal

mixture of ammonium and nitrate is required for maximum cell density (AB axis). Little effect of varying mixtures of ammonium and sodium are seen, and in the absence of nitrate only poor cell density is seen (AD axis). These effects are only manifested at the high (20 mM) concentration of ions as identical mixture surfaces at the low ion concentration (2 mM) show very little changes in contour with variations in proportions (Figure 4C). These results suggest that overall charge may be a factor here. Thus, in the absence of one cation, equal parts of the other cation and an anion are required. When no anion at all is present, things grow poorly, perhaps as much due to the charge difference as to the lack of nitrate as a nutrient. These effects are examined further below in section 3.4.

Interesting insights can be gained by examining a few other variations in mixture components. For example, when ammonium, nitrate and phosphate, the major macronutrients for algal growth, were varied at constant sodium and potassium in the absence of chloride (Fig 5A), the preference for nitrate over ammonium can be very clearly seen (AB axis). While varying the proportions of phosphate and ammonium in the absence of nitrate had very little effect on cell density, which remained low (CA axis), changes in the relative proportions of phosphate and nitrate had a great effect on biomass which was highest when nitrate was highest and phosphate was lowest (CB axis, no ammonium). Similarly, it was previously observed that the N:P ratios for optimal growth rates of different mixtures are highly dependent upon the ionic context (Evens and Niedz 2010).

Holding ammonium constant at zero with intermediate levels of sodium and potassium allows the effects of varying proportions of chloride and phosphate on cell density to be seen. These had been predicted from the Piepel plots to be important. As indicated in Fig

5B, chloride and phosphate were both antagonistic with nitrate for biomass production. In addition, it can clearly be seen that there were very little interactive effects between chloride and phosphate independent of the nitrate concentration. Finally, how the principal players, nitrate, sodium, and potassium, interact can clearly be seen in Fig. 5C which shows mixtures of these factors at low phosphate (0.01 mM) and the absence of ammonium and chloride. Here it can be seen that maximal cell density is obtained at a roughly equal mix of these three factors. Changes of this surface with different (lower) total ion concentrations suggest that even higher $OD_{600}$s might be achievable with the same relative proportions of the varied factors at total ion concentrations above 20 mM.

*3.4 Analysis of lipid production by mixtures showing good growth*

It was of interest to examine the number of mixtures showing good growth, the mixtures that led to high cellular lipid contents, and, most importantly, mixtures which led to high overall lipid productivity. The number of mixtures supporting good growth decreased logarithmically at higher and higher ODs. A final $OD_{600}$ of 0.7 was arbitrarily chosen here as providing sufficient growth to be of interest in production. Thirty-seven unique mixtures supported growth to this OD or above, twelve of which supported growth to $OD_{600} \geq 1.0$ (Fig. 6A). Although there is some variation in the composition of the different mixtures supporting good growth, in general their characteristics were as determined by RSM as described above (Section 3.3). Interestingly, when this criterion is used, this *Chlorella* strain does not appear to prefer one nitrogen source over the other as eleven of the 37 mixtures contained only $NO_3^-$ whereas nine contained only $NH_4^+$.

Next, these same mixtures were examined for their effect on cellular lipid content, assessed by FL1 fluorescence as determined by flow cytometry of BODIPY® stained cells. Surprisingly, a very wide variation (over 50 fold for the two extreme cases, mixtures 75 and 106) in lipid content was observed (Table 1 and Fig. 6B). Eight unique mixtures gave a mean FL1 of 50,000 or above (Table 1 and Fig. 6B). Curiously, although it is commonly assumed and often observed that lipid content increases in nitrogen-deficient media, the total nitrogen content of these mixtures varied from 0.55 to 13 mM (Table 1). Consequently, there appears to be little correlation here between the nitrogen content of the medium and final lipid content. Furthermore, there was no correlation with the N/P ratio as this varied over 400 fold (from 0.15 to 64) in the different mixtures giving high mean FL1 (Table 1). Thus, a low N/P ratio alone cannot explain the high lipid content.

Finally, in terms of potential biodiesel production, total lipid production, i.e. the product of cellular lipid content and total cell numbers, is the most important variable. This was ascertained here by multiplying cellular lipid content (mean FL1 values) by $OD_{600}$, a reasonable proxy for cell numbers. By this measure a number of mixtures gave high lipid productivities (Fig 6C), with the five highest producers (43, 44, 70, 80, 106) not surprisingly found amongst the mixtures giving the highest mean lipid content (Table 1). Accordingly, these mixtures define the composition space within which further R&D might produce the best trade-off between total biomass production and high cellular lipid content.

*3.5 Curious cases and oddities.*

Finally, the highly varied ionic composition used allows a number of interesting and unexpected observations to be made. It should be noted that many of these would not be seen with the different media formulations currently in use. Since the method used here was explicitly set up to avoid confounding ions, the anions and cations that were varied used either $H^+$ or $OH^-$ as counter ion, and no pH control or buffering was attempted or indeed possible. This means that the initial pH of many of the resulting solutions was either highly acidic (56 < pH 3) or highly basic (26 > pH10) (Figure S2). However, there was a great difference in the behavior at the two different pH extremes. An initial acidic pH was very unfavorable for growth with only 5/56 (9%) supporting growth above an $OD_{600}$ of 0.2. On the other hand, an initial alkaline pH does not appear to be unfavorable since 26/26 (100%) of the mixtures with an initial pH > pH10 supported growth above an $OD_{600}$ of 0.2, and in fact, 14/26 (54%) had a final $OD_{600}$ above 0.7. This is surprising in that *Chlorella* are not noted for being particularly basophilic. Similar effects of different initial pHs on growth rates have been previously noted (Evens and Niedz 2010).

The Scheffe Mix Model used here for unconfounded ion mixtures also allowed the examination of specific cation and anion requirements, in particular $Na^+$, $K^+$, and $Cl^-$, almost universally found in algal media. To our knowledge, freshwater microalgae have never been probed for their requirements for these ions. Many media formulations contain $Cl^-$ merely because HCl is added to adjust the pH. Here, this was not the case, and 117 of the 210 solutions contained no $Cl^-$. Very surprisingly, this strain grew very well in the absence of $Cl^-$ with 23 unique mixtures giving growth to an $OD_{600}$ of 0.8 or better and 9 unique mixtures supporting growth to an $OD_{600}$ of 1.0 or better (Table 2). In fact, as noted

above (Piepel traces, Figure 2), $Cl^-$ appears to inhibit growth and the highest $OD_{600}$s were obtained in its absence. This is in accordance with a previous study which concluded that *C. vulgaris* demonstrates a positive response to cations in its growth medium, but a negative response to anions (Evens and Niedz 2010).

Next, requirements for the important cations $Na^+$ and $K^+$ were examined. Of the 113 mixtures that contained no $Na^+$, 19 unique mixtures supported growth to an $OD_{600} > 0.5$ with nine unique mixtures supporting growth to an $OD_{600} > 0.7$ (Table 3). It is noteworthy that these nine mixtures all contained no $Cl^-$, establishing an apparent requirement for the absence of this anion in the absence of $Na^+$. Of the 105 mixtures where $K^+$ was absent, there were 22 unique mixtures which supported growth to an $OD_{600} > 0.5$, with ten unique mixtures supporting growth to an $OD_{600} > 0.7$ (Table 3). Similar to what was seen with $Na^+$, the majority of these mixtures (80%) contained no $Cl^-$. Not surprisingly, even fewer good mixtures were found when the absence of both $Na^+$ and $K^+$ cations was probed. There were forty-two unique mixtures which lacked both cations, but only 21 of these were even capable of supporting growth to an $OD_{600} > 0.2$, and only 4 could support growth above an $OD_{600}$ of 0.4 (Table 3). Interestingly, three of these also lacked $Cl^-$. Growth of microalgae on media simultaneously lacking $Na^+$, $K^+$, and $Cl^-$ as major ions has not been previously observed.

*4.0 Conclusions*

Here, an unconfounded Scheffe Mix approach and RSM with *Chlorella sp.* EN1234 showed that the major media anions $PO_4^-$ and $Cl^-$ negatively influence final cell densities.

Maximal cell density is obtained with nitrate over ammonium. Several mixtures lacking $Na^+$, $K^+$, and $Cl^-$ as major ions supported significant growth. Little correlation between nitrogen content and total lipid content was found with mixtures that supported high lipid productivity. Thus, these mixtures define the composition space within which further investigation might produce the best trade-off between total biomass production and high cellular lipid content.

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**Figure legends**

**Figure 1. 23S rRNA tree of *Chlorella sp.* EN1234**

Distances of *Chlorella sp.* EN1234 to related species were determined as described in Materials and Methods.

**Figure 2. Peipel Plot of *Chlorella sp.* EN1234 growing on mixtures containing both $NH_4^+$ and $NO_3^-$**

Actual component and factor coding was used with Design Expert (DX9) to generate Piepel plots for the mixtures with *Chlorella sp.* EN1234. The components were: A: $NH_4^+$, 0.119; B: $NO_3^-$, 0.119; C: $PO_4^-$, 0.198; D $Na^+$, 0.188; E: $K^+$, 0.188; F: $Cl^-$, 0.188. Traces were done at different total ion concentrations of: A, 20 mM; B, 11 mM; C, 2 mM

**Figure 3. Peipel Plot of *Chlorella sp.* EN1234 centered around the best run (Run 44).**

Actual component and factor coding was used with Design Expert (DX9) to generate Piepel plots for *Chlorella sp. EN1234* centered around the best run (highest observed $OD_{600}$). The components were: A: $NH_4^+$, 0.000; B: $NO_3^-$, 0.377; C: $PO_4^-$, 0.010; D: $Na^+$, 0.312; E: $K^+$, 0.301; F: $Cl^-$, 0.000. Traces were done at different total ion concentrations of: A, 20 mM; B, 11 mM; C, 2 mM

**Figure 4. 3D Surface Plots of *Chlorella sp.* EN1234 at constant $PO_4^-$ (0.01 mM)**

A. The response of cell density to changes in [$NH_4^+$] (A), [$NO_3^-$] (B), and [$K^+$] (E) at constant $Na^+$ (0.312), $PO_4^-$ (0.01 mM), and $Cl^-$=0. Total ion = 20 mM

B. The response of cell density to changes in [$NH_4^+$] (A), [$NO_3^-$] (B), and [$Na^+$] (C) at constant $K^+$ (0.301), $PO_4^-$ (0.01 mM), and $Cl^-$=0. Total ion = 20 mM

C. The response of cell density to changes in $[NH_4^+]$ (A), $[NO_3^-]$ (B), and $[K^+]$ (C) at constant $Na^+$ (0.312), $PO_4^-$ (0.01 mM), and $Cl^-$=0. Total ion = 2 mM

**Figure 5. 3D Surface Plots of *Chlorella sp.* EN1234 with varying $PO_4^-$ or $K^+$**

A. The response of cell density to changes in $[NH_4^+]$ (A), $[NO_3^-]$ (B), and $[PO_4^-]$ (C) at constant $Na^+$ (0.312), $K^+$ (0.301), and $Cl^-$=0. Total ion = 20 mM

B. The response of cell density to changes in $[NO_3^-]$ (B), $[PO_4^-]$ (C), and $[Cl^-]$ (F) at constant $NH_4^+$ = 0, $Na^+$ (0.312), and $K^+$ (0.251). Total ion = 20 mM

C. The response of cell density to changes in $[NO_3^-]$ (B), $[Na^+]$ (D), and $[K^+]$ (E) at constant $NH_4^+$ = 0, $PO_4^-$ (0.010), and $Cl^-$=0. Total ion = 20 mM

**Figure 6. Final $OD_{600}$s, mean lipid per cell, and total lipid productivity of selected mixtures**

The responses of mixtures producing a final $OD_{600}$ of 0.8 or greater are shown. (A) Final $OD_{600}$, (B) Cellulare lipid content as determined by FL1 fluorescence using flow cytometry, (C) total lipid productivity.

Table

## Table 1 Unique Mixtures Giving High Final Lipid Content

| Mixture | $OD_{600}$ | Mean FL1 | N/P | Total N mM | $NH_4^+$ mM | $NO_3^-$ mM | $PO_4^-$ mM | $Na^+$ mM | $K^+$ mM | $Cl^-$ mM | total ion mM |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 43 | 0.91 | 74100 | 0.16 | 1 | 0.57 | 0.43 | 6.33 | 6.42 | 0 | 6.24 | 20 |
| 44 | 1.18 | 70800 | 1.11 | 0.73 | 0 | 0.73 | 0.66 | 0 | 0.61 | 0 | 2 |
| 59 | 0.78 | 71700 | 5 | 0.55 | 0.24 | 0.31 | 0.11 | 3.28 | 3.64 | 3.43 | 11 |
| 80 | 1.56 | 98300 | 65.2 | 13.0 | 6.32 | 6.71 | 0.2 | 6.15 | 0.61 | 0 | 20 |
| 110 | 0.94 | 155000 | 2.12 | 2.29 | 0.73 | 1.56 | 1.08 | 2.61 | 0.98 | 0.099 | 7.07 |
| 148 | 0.83 | 53800 | 63.0 | 1.28 | 0.66 | 0.62 | 0.02 | 0.66 | 0.039 | 0.0080 | 2 |
| 200 | 0.72 | 76100 | 0.91 | 3.42 | 3.422 | 0 | 3.75 | 3.83 | 0 | 0 | 11 |
| 201 | 0.81 | 84700 | 5 | 1 | 1 | 0 | 0.2 | 18.8 | 0 | 0 | 20 |

**Table 2 Mixtures with no $Cl^-$ giving good growth**

| Mixture | pH | $OD_{600}$ | $NH_4^+$ mM | $NO_3^-$ mM | $PO_4^-$ mM | $Na^+$ mM | $K^+$ mM | $Cl^-$ mM | [total ion] mM |
|---|---|---|---|---|---|---|---|---|---|
| 78 | 11.4 | 1.05 | 0 | 0.55 | 0.11 | 5.03 | 5.31 | 0 | 11 |
| 96 | 3.73 | 1.09 | 0 | 1.03 | 0.02 | 0 | 0.95 | 0 | 2 |
| 192 | 11.25 | 1.06 | 3.42 | 0 | 3.75 | 3.83 | 0 | 0 | 11 |
| 210 | 9.46 | 1.11 | 0 | 0.1 | 0.02 | 0.94 | 0.94 | 0 | 2 |
| 44 | 10.72 | 1.18 | 0 | 0.73 | 0.665 | 0 | 0.61 | 0 | 2 |
| 123 | 7.6 | 1.20 | 0.68 | 0 | 0.02 | 0.66 | 0.63 | 0 | 2 |
| 207 | 9.54 | 1.37 | 6.50 | 6.16 | 0.57 | 0 | 6.78 | 0 | 20 |
| 90 | 9.49 | 1.46 | 6.32 | 6.71 | 0.2 | 6.15 | 0.61 | 0 | 20 |
| 4 | 4.0 | 1.55 | 0.073 | 0.076 | 0.66 | 0.59 | 0.60 | 0 | 2 |

**Table 3 Mixtures without $Na^+$ and mixtures without $K^+$ giving good growth**

| Mixture | $OD_{600}$ | $NH_4^+$ mM | $NO_3^-$ mM | $PO_4^-$ mM | $Na^+$ mM | $K^+$ mM | $Cl^-$ mM | [total ion] mM |
|---|---|---|---|---|---|---|---|---|
| **No $Na^+$** | | | | | | | | |
| 44 | 1.18 | 0 | 0.73 | 0.66 | 0 | 0.61 | 0 | 2 |
| 38 | 0.873 | 11.7 | 0 | 8.30 | 0 | 0 | 0 | 20 |
| 96 | 1.13 | 0 | 1.03 | 0.02 | 0 | 0.95 | 0 | 2 |
| 111 | 0.92 | 0 | 5.71 | 0.11 | 0 | 5.18 | 0 | 11 |
| 141 | 0.71 | 0.77 | 0.0077 | 0.54 | 0 | 14.2 | 0 | 15.5 |
| 145 | 0.86 | 0.67 | 0.62 | 0.036 | 0 | 0.67 | 0 | 2 |
| 162 | 1.47 | 6.50 | 6.16 | 0.57 | 0 | 6.78 | 0 | 20 |
| 186 | 0.82 | 0 | 0.1 | 0.02 | 0 | 1.88 | 0 | 2 |
| 191 | 0.79 | 19.8 | 0 | 0.2 | 0 | 0 | 0 | 20 |
| **No $K^+$** | | | | | | | | |
| 175 | 0.70 | 1 | 0 | 0.2 | 18.8 | 0 | 0 | 20 |
| 202 | 0.75 | 0.43 | 0.12 | 3.54 | 3.44 | 0 | 3.47 | 11 |
| 199 | 0.83 | 3.42 | 0 | 3.75 | 3.83 | 0 | 0 | 11 |
| 74 | 0.86 | 0.95 | 0 | 0.02 | 1.03 | 0 | 0 | 2 |
| 61 | 0.87 | 11.7 | 0 | 8.30 | 0 | 0 | 0 | 20 |
| 70 | 0.89 | 0.57 | 0.43 | 6.33 | 6.42 | 0 | 6.24 | 20 |
| 77 | 0.91 | 0 | 5.16 | 0.11 | 5.73 | 0 | 0 | 11 |
| 120 | 0.96 | 5.60 | 0 | 0.11 | 5.29 | 0 | 0 | 11 |
| 181 | 0.98 | 0 | 9.82 | 0.2 | 9.98 | 0 | 0 | 20 |
| 191 | 0.79 | 19.8 | 0 | 0.2 | 0 | 0 | 0 | 20 |
| **No $Na^+$ and $K^+$** | | | | | | | | |
| 204 | 0.415 | 1.98 | 0 | 0.02 | 0 | 0 | 0 | 2 |
| 169 | 0.5445 | 0.1 | 0 | 0.02 | 0 | 0 | 1.88 | 2 |
| 191 | 0.79 | 19.8 | 0 | 0.2 | 0 | 0 | 0 | 20 |
| 38 | 0.873 | 11.7 | 0 | 8.30 | 0 | 0 | 0 | 20 |

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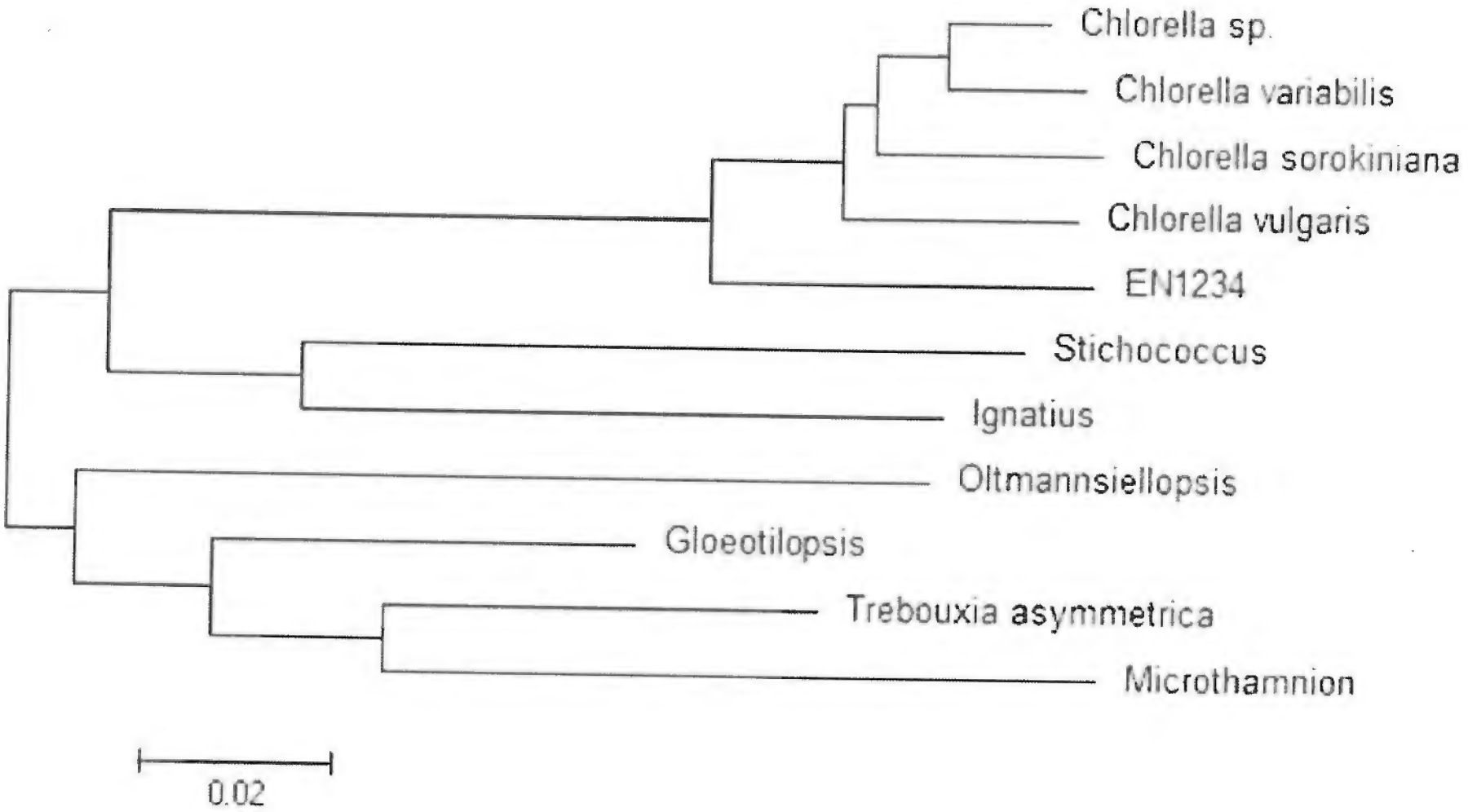

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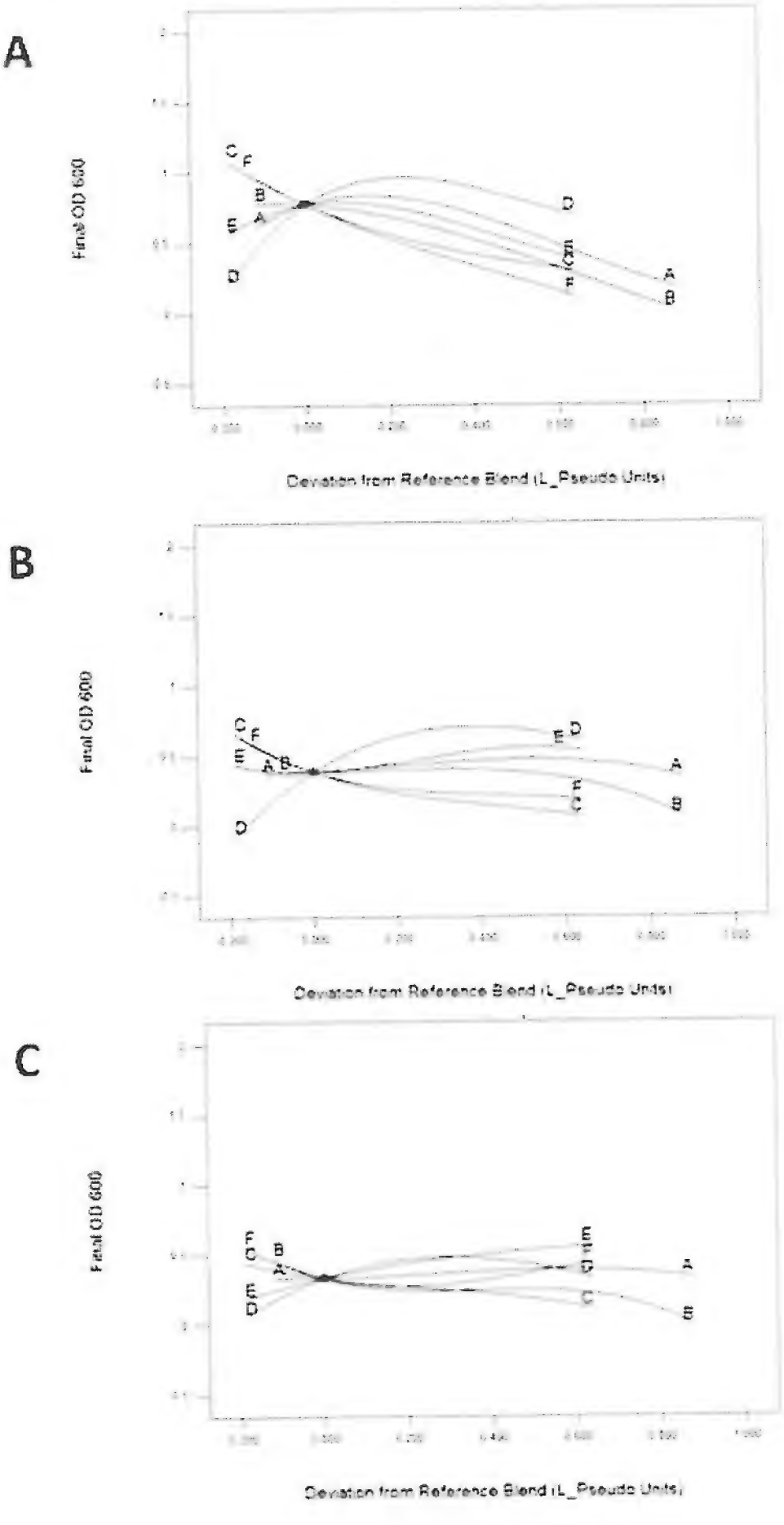

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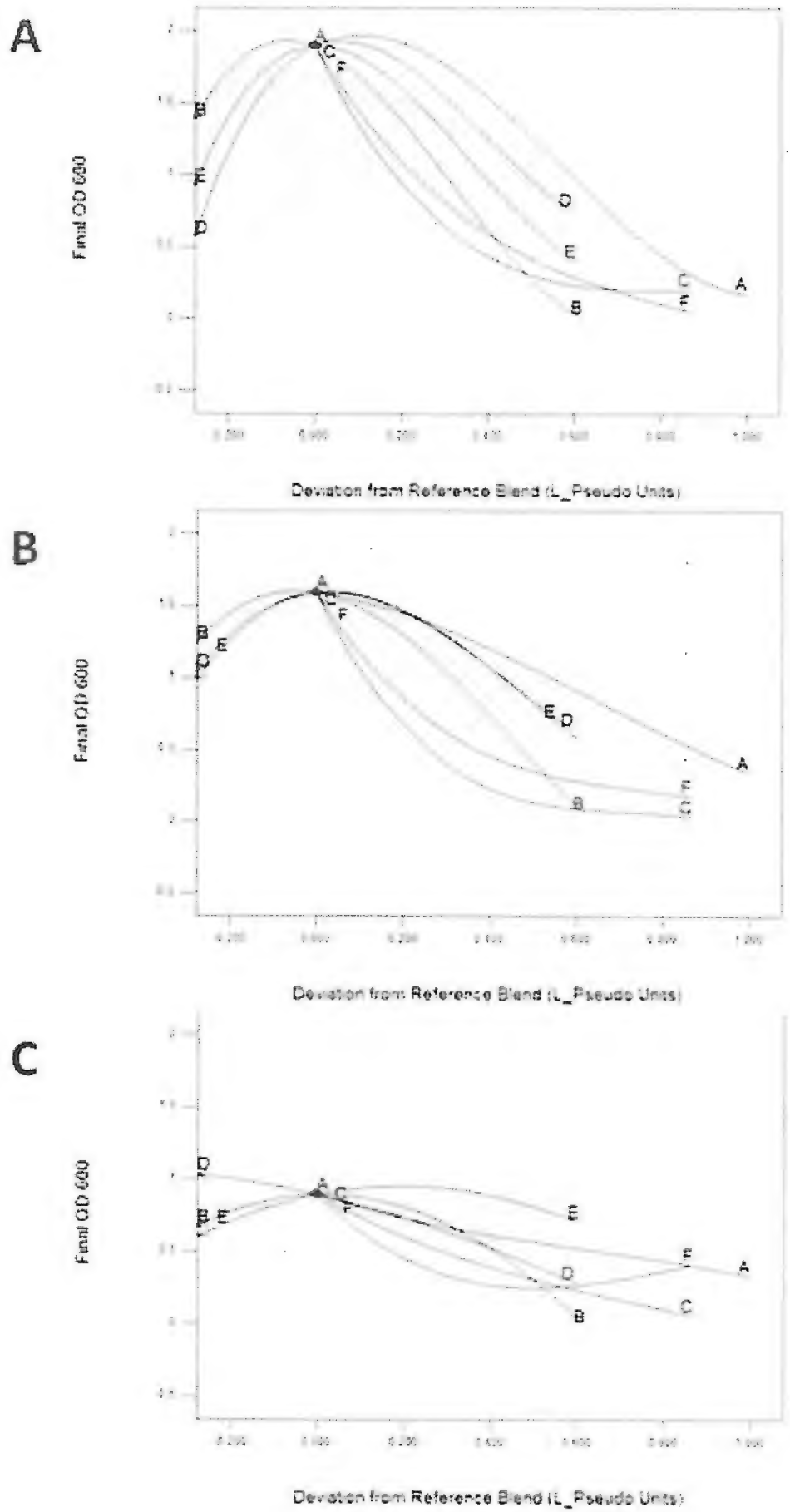

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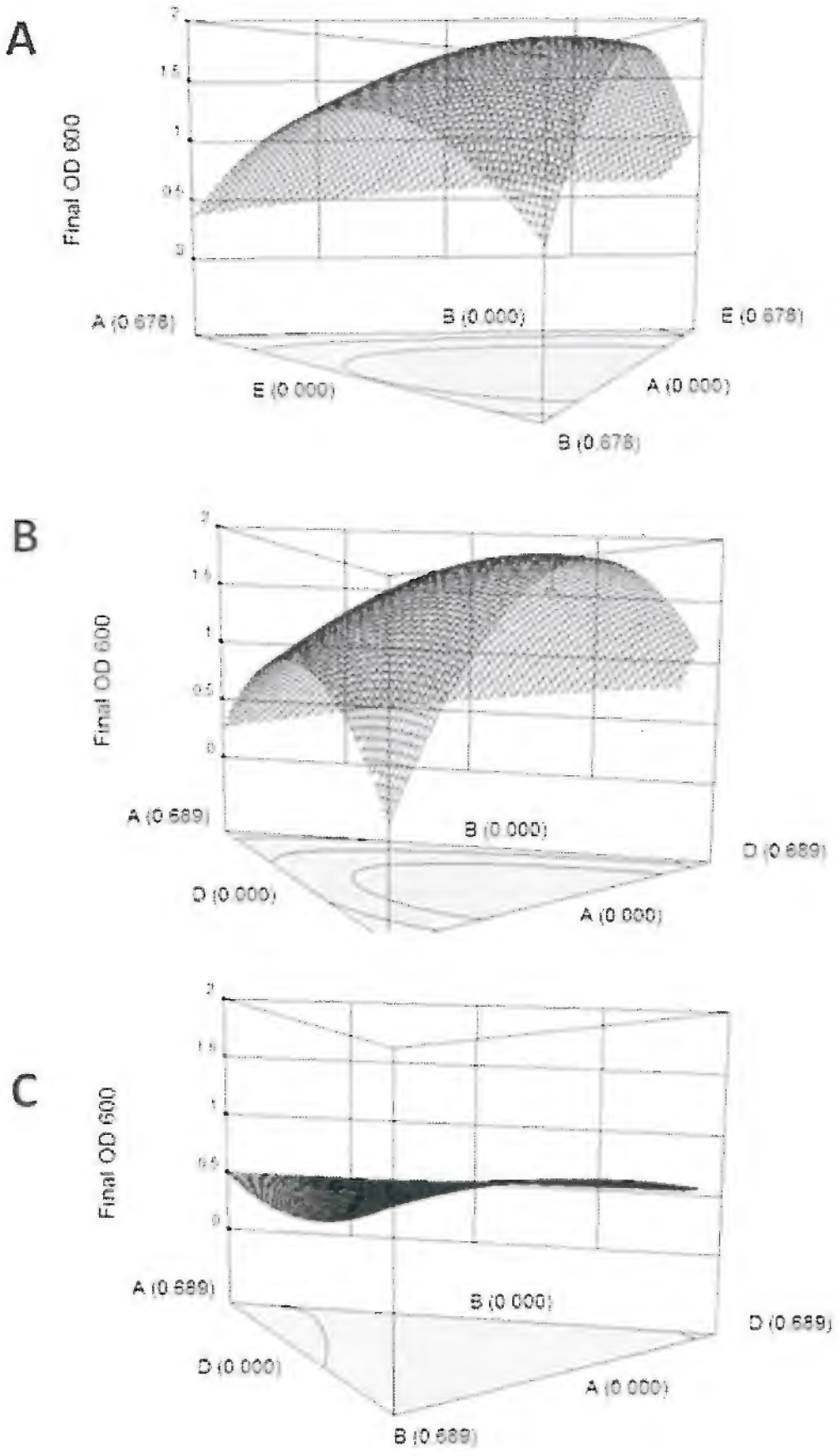

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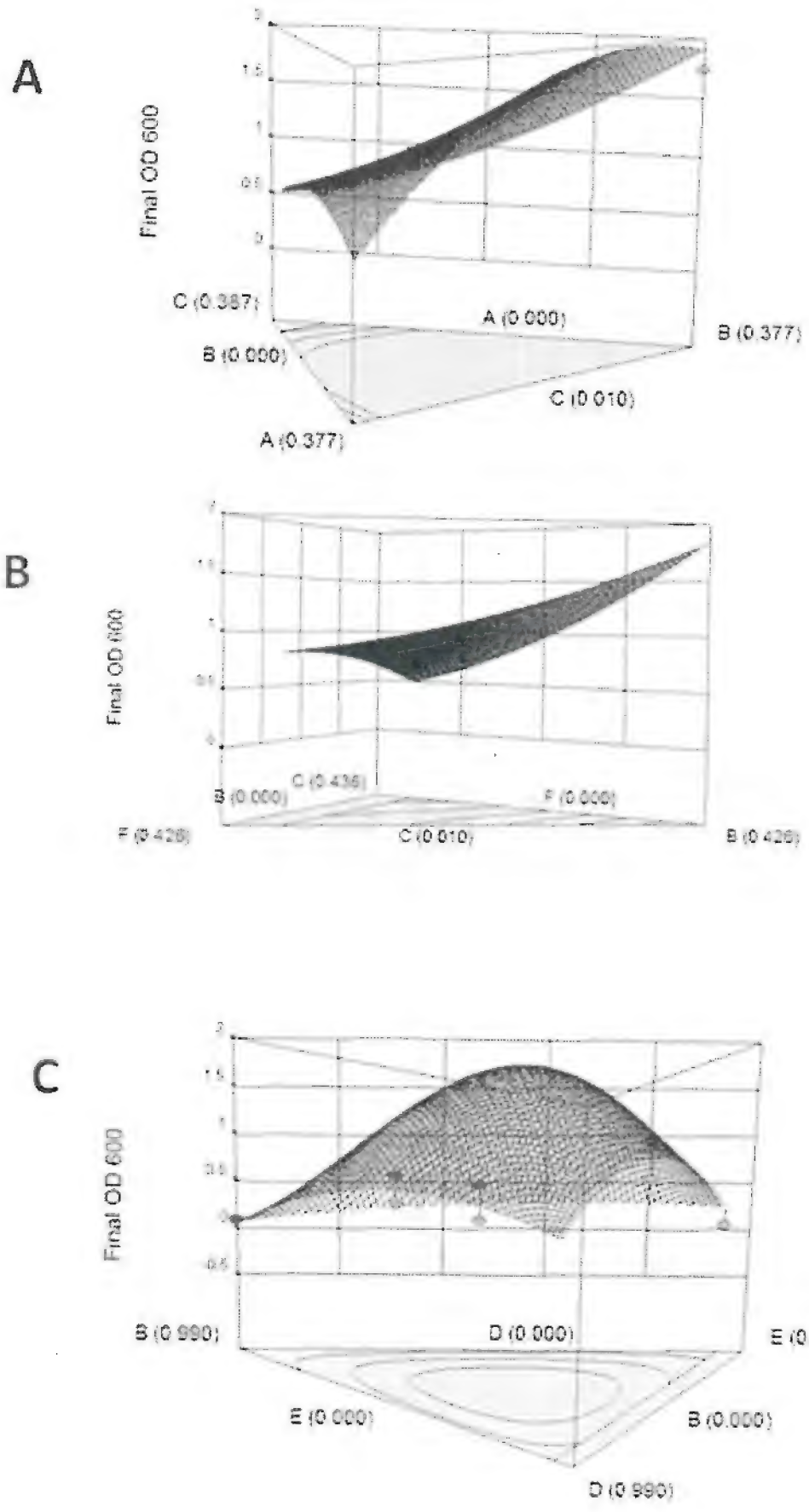

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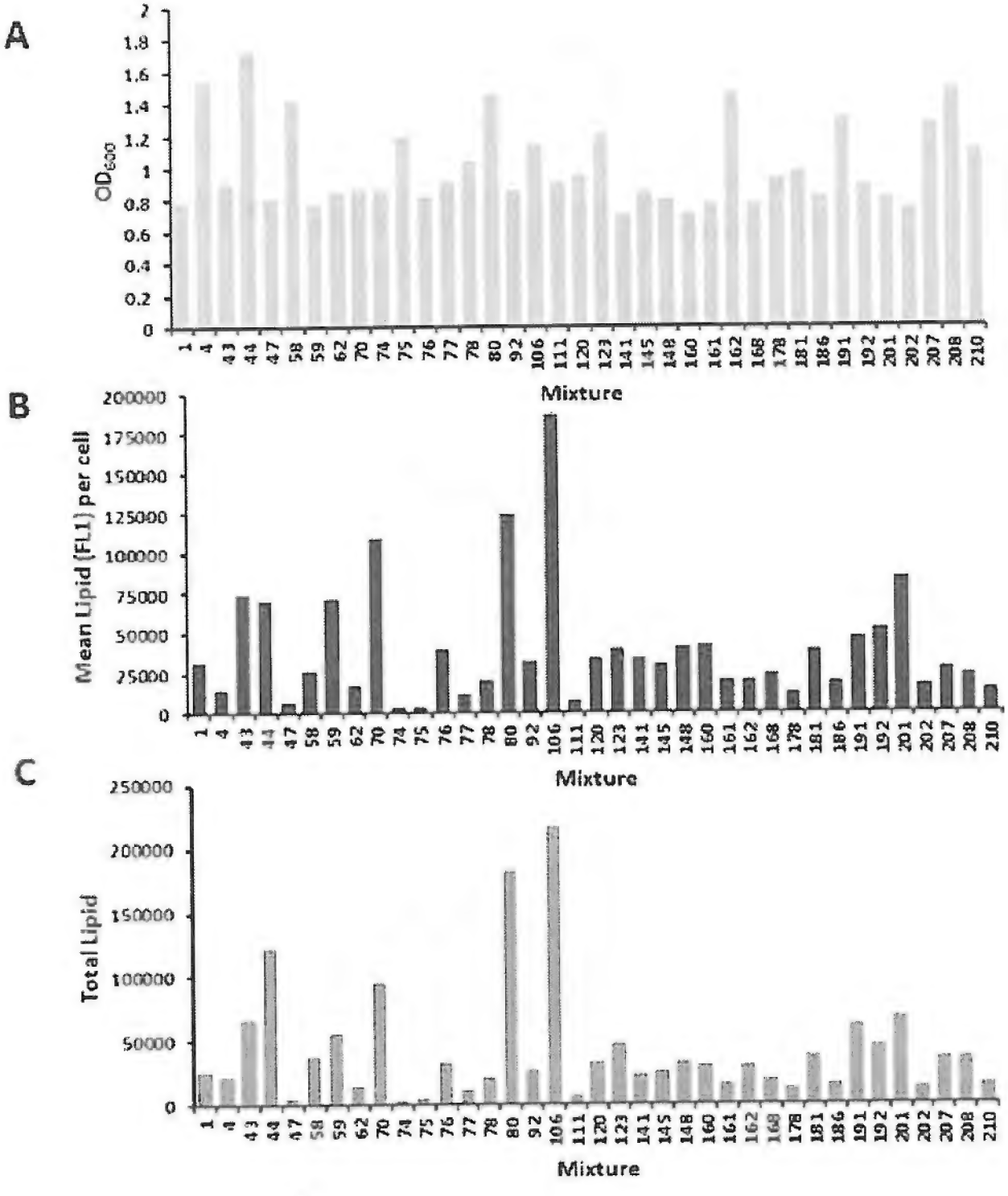

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